Medium-Chain-Length Poly(β-Hydroxyalkanoate) Synthesis from Triacylglycerols by *Pseudomonas saccharophila*

Abstract. *Pseudomonas saccharophila* NRRL B-628 is capable of utilizing agricultural lipids for growth. The organism exhibited good growth with triacylglycerol substrates that contained saturated fatty acyl moieties such as coconut oil (CO; C_{10-12} fatty acids) and tallow (T; C_{16-18} fatty acids). Electron micrographs of the triacylglycerol-grown cells showed the presence of intracellular granules indicative of poly(β-hydroxyalkanoate) (PHA) production. Cells grown in a 250-ml CO-containing medium produced ca. 0.2 g of medium-chain-length (mcl)-PHA. Gas chromatographic analysis showed that β-hydroxyoctanoic acid (30%), β-hydroxydecanoic acid (40%), and β-hydroxydodecanoic acid (16%) were the major monomer repeat-units of the CO-derived polymer. The estimated mean molecular mass of the CO-derived mcl-PHA as determined by gel permeation chromatography was 13.1×10^4 g/mol with a polydispersity of 3.16.

Poly(β-hydroxyalkanoates) (PHA) are biopolymers synthesized by numerous microorganisms in response to carbon excess but otherwise nutrient-limiting conditions [18]. PHA polymers are potentially useful as biodegradable substitutes for petroleum-derived thermoplastics and elastomeric materials [1, 16]. Among PHA-producing organisms, the pseudomonads grown on alkanoic acids specifically synthesize a class of PHAs that contain only the medium-chain-length β-hydroxyalkanoic acids of C_{6-14} carbon atoms [4, 8, 11]. These PHAs are particularly suited for the development of elastomeric materials [1].

The accumulation of mcl-PHA by pseudomonads occurs in the presence of carbon substrates such as alkanes, alkenes, fatty acids, and carbohydrates [8, 10, 13, 14]. Cromwick et al. [6] first reported the production of mcl-PHA from an intact triacylglycerol substrate by *Ps. resinovorans*. Subsequently, Ashby and Foglia [2] documented the utilization of a spectrum of agricultural triacylglycerols by this pseudomonas species for mcl-PHA accumulation. In this communication, we report on our investigation of the synthesis of mcl-PHA from triacylglycerols by another organism, namely *Ps. saccharophila*. Hou and Johnson [12] previously reported that this species contained a high lipase activity. The microorganism was also shown to accumulate high-molecular-mass PHA [15, 19]. These properties could be exploited for the development of an efficient triacylglycerol fermentation system for mcl-PHA production.

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Materials and Methods

Bacterium and growth conditions. *Ps. saccharophila* NRRL B-628 was obtained from the U.S. Department of Agriculture, ARS, National Center for Agricultural Utilization Research (Peoria, IL). The stock cultures were prepared and maintained in Luria (L) medium (1%, wt/vol, tryptone; 0.5%, wt/vol, yeast extract; 0.5%, wt/vol, NaCl). Cells were grown in an incubator-shaker set at 30°C and 250 rpm.

Cell growth with triacylglycerols. The chemically defined medium E* containing a limiting nitrogen source [4] was supplemented with 0.5% (wt/vol) of a test triacylglycerol substrate as the sole carbon source. The composition of E* medium has been previously detailed [4]. The medium (250 ml in a 500-ml Erlenmeyer flask) was inoculated with 1 ml of an overnight culture of *Ps. saccharophila* in L medium. Cells were grown at 30°C with shaking at 250 rpm. Aliquots of the culture were withdrawn at various time intervals, and the viable cell numbers were determined by serial-dilution plating of the samples on solid (1%, wt/vol agar) L medium. Tallow was obtained from Miniat Inc. (Chicago,

IL); soybean oil under the trade name of Weston was purchased from a local supermarket; sunflower oil was supplied by Eastern Regional Research Center's Dairy Pilot Processing Plant; and coconut oil was purchased from Sigma/Aldrich (St. Louis, MO). Chemicals were procured from Sigma/Aldrich.

PHA production and analysis. One milliliter of an overnight culture of *Ps. saccharophila* in L medium was added to 250 ml of E* medium supplemented with 0.5% (wt/vol) of a triacylglycerol substrate. Cells were grown at 30°C with 250-rpm shaking for 48–72 h. An aliquot of the 3-day culture was withdrawn for viewing of PHA inclusion bodies by electron microscopy. PHA polymer was isolated from the batch cultures by a modified version of the chloroform extraction procedure described by Cromwick et al. [6]. The polymer was extracted by stirring the lyophilized cells in chloroform at room temperature for ≥16 h. Polymer was precipitated by drop-wise addition of the chloroform extracts into pre-chilled methanol. The monomer repeat-unit composition of the polymer was determined by acid hydrolysis of the PHA, followed by gas chromatography/mass spectroscopic analysis of the methylated β-hydroxy acid monomers [6]. The mean molecular mass of the polymer was estimated by gel permeation chromatography [2], by use of polystyrene standards (Polyscience Corporation, Warrington, PA) with a low polydispersity.

Electron microscopy study. Cells were treated with 1% glutaraldehyde. Post-fixation treatment was performed in a 2% osmium tetroxide solution. Embedment was effected by sequential incubations in propylene oxide, a 1:1 (vol/vol) propylene oxide/epoxy resin mixture, and finally in a fresh epoxy solution. Thin sections (50–60 nm) were prepared and stained with lead citrate and uranyl acetate solutions. Electron micrographs were obtained on a Phillips Model CM12 electron microscope operated in the bright field imaging mode and captured on Kodak Type 4489 film at 10,000× magnification.

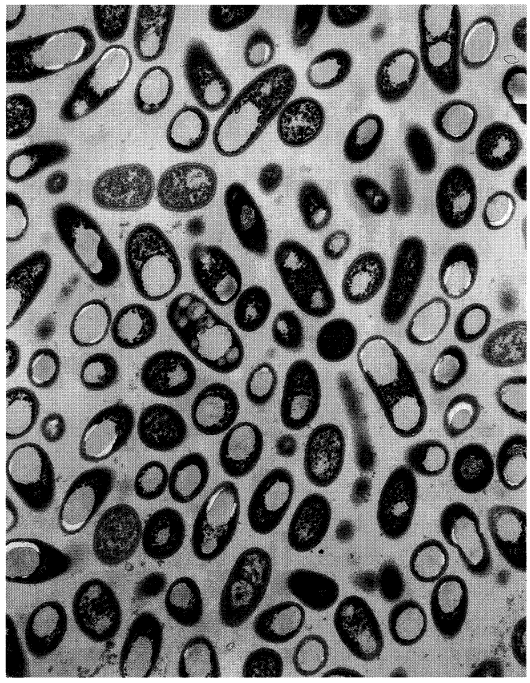

Fig. 1. Thin-section electron microscopic images of *Ps. saccharophila* NRRL B-628. Cells were grown in E* medium supplemented with 0.5% (wt/vol) tallow. Incubation was performed in a 30°C incubator-shaker operated at 250 rpm with rotary shaking. Cells were harvested by centrifugation and processed for electron microscopic study after a 72-h growth.

Results and Discussion

We examined the growth of *Ps. saccharophila* NRRL B-628 in E* medium containing one of the following triacylglycerols: tallow (T), soybean oil (SB), sunflower oil (SF), and coconut oil (CO). Growth in medium containing oleic acid (OL) was also monitored for comparison. Culture medium was inoculated with the bacterium at a concentration of 5.0–6.5 $\times$ 10^6 cells/ml. After an overnight incubation (16 h), all cultures grew to a density of 0.5–3.5 $\times$ 10^9 cells/ml. At this point, the best and poorest cell growth was observed with cultures supplemented with CO and SB, respectively. Subsequent to the 16-hour growth, cells in OL-, SB-, or SF-containing medium suffered viability loss of up to an order of magnitude for the next 32 h. Those cells incubated in medium containing T or CO, however, continued to grow to a concentration of 1.3 $\times$ 10^{10} (T) and 2.2 $\times$ 10^{11} (CO) cells/ml. An additional 24-h incubation resulted in a decrease in viable-cell counts with the OL- (4.5 $\times$ 10^8 cells/ml) and CO- (1.8 $\times$ 10^{10} cells/ml) containing cultures. However, increased cell numbers were observed with cultures grown in SB (1.2 $\times$ 10^9 cells/ml), SF (3.4 $\times$ 10^8 cells/ml), and T (1.8 $\times$ 10^{11} cells/ml). The data showed that *Ps. saccharophila* grew well in triacylglycerols that contained saturated fatty acyl components. For example, coconut oil, which contains lauric acid (C_{12}) as its major (47%) fatty acid component [5], supported *Ps. saccharophila* growth to a high density within 48 h of incubation. On the other hand, the soybean and sunflower oils [7, 17], with their high contents (50–75%) of unsaturated linoleoyl ($C_{18:2}$) and oleoyl ($C_{18:1}$) groups, gave lower density cell growth after 72-hour incubation.

When the triacylglycerol-grown cells were viewed under an oil-immersion, phase-contrast microscope, small amounts of intracellular refractile bodies could be seen (data not shown). Figure 1 shows a representative electron micrograph of the thin sections of triacylglycerol-grown *Ps. saccharophila*. Approximately 80% of these cells contained granular structures characteristic of PHA inclusion bodies [19]. In contrast to the observation by Young et al. [19] with sucrose-grown cells, our results indicate that *Ps. saccharophila* growing on triacylglycerol substrates continued to produce the PHA granules even after an extended growth period.

We next performed a small-scale isolation of PHA with a 250-ml culture of triacylglycerol-grown *Ps. saccharophila*. Small amounts (ca. 0.2 g total yield) of the biopolymer were obtained from CO-grown cells. The

Table 1. Monomer composition of PHA produced by *Ps. saccharophila* and *Ps. saccharophila* grown in coconut oil

β-Hydroxyalka(e)noyl methyl ester[b]	mol-%[a]	
	Ps. saccharophila	*Ps. resinovorans*[c]
$C_{6:0}$	6	8
$C_{8:0}$	30	37
$C_{10:0}$	40	35
$C_{12:0}$	16	17
$C_{12:1}$	1	N.D.
$C_{14:0}$	4	3
$C_{14:1}$	2	N.D.
$C_{14:2}$	1	N.D.

[a] mol-% was calculated from the integrated areas of the chromatography peaks.

[b] Cells were grown in E* medium supplemented with coconut oil (0.5%, wt/vol) at 30°C with shaking at 250 rpm for 3 days. PHA was isolated and processed for GC/MS analysis as described in Materials and Methods.

[c] Data were from Ashby and Foglia [2]. N.D., not detected.

results of the PHA monomer repeat-unit analysis showed that the major β-hydroxy acids of the polymer were β-hydroxyoctanoic acid and β-hydroxydecanoic acid (Table 1). The polymer produced by *Ps. saccharophila* on coconut oil is classified as an mcl-PHA. It is noted that the *Ps. saccharophila* PHA monomer composition is different from that obtained from the PHA of CO-grown *Ps. resinovorans* [2]. Unlike the *Ps. resinovorans* polymer, the mcl-PHA produced by *Ps. saccharophila* grown on coconut oil contained a higher ratio of C_{10} to C_8 repeat-unit. Furthermore, while <1% unsaturated monomers were detected in the *Ps. resinovorans* PHA [2], these olefinic repeat-units made up about 4% of the monomers in the *Ps. saccharophila* polymer. Gel permeation chromatographic analysis of the *Ps. saccharophila*-produced mcl-PHA indicated that its mean molecular mass (M_n) was 13×10^4 g/mol with a polydispersity of 3.16. In contrast, the corresponding PHA from *Ps. resinovorans* had a slightly lower molecular mass and polydispersity [2].

This study showed that *Ps. saccharophila* is capable of growing in culture medium supplemented with triacylglycerol substrates. We also showed that this microorganism is capable of producing the mcl-PHA. Earlier studies had indicated that *Ps. saccharophila* grown on carbohydrate substrates could produce the short-chain-length poly(β-hydroxybutyrate) (PHB), but no monomer composition data were presented [9, 15]. In view of the results presented in this paper, it appears that this bacterium possesses PHA-synthesis pathway(s) that allow it to produce either the short-chain-length (C_4)- or the mcl (C_{8-10})-PHAs depending on the available substrate. This property, together with the ability of *Ps. saccharophila* to grow well on other industrially important substrates such as starch and gluconate [3], should allow the development of a mixed-substrate fermentation process to produce novel PHAs.

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